

# Biosynthesis of indole-3-acetic acid via the indole-3-acetamide pathway in *Streptomyces* spp.

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**Various *Streptomyces* spp. including *S. violaceus*, *S. scabies*, *S. griseus*, *S. exfoliatus*, *S. coelicolor* and *S. lividans* secrete indole-3-acetic acid (IAA) when fed with L-tryptophan (Trp). Production of IAA was detected in *Streptomyces* strains causing potato scab as well as in non-pathogenic strains. The pathways for IAA synthesis from Trp were investigated in *S. violaceus* and *S. exfoliatus*. Indole-3-acetamide (IAM), indole-3-lactic acid (ILA), indole-3-ethanol (IEt) and IAA were identified by HPLC and GC-MS. *Streptomyces* cells were capable of catabolizing IAM, ILA, IEt and indole-3-acetaldehyde (IAAld) into IAA. Incorporation of radioactivity into IAM, IAA and IAL but not IEt was detected when cells were fed with L-[3-<sup>14</sup>C]tryptophan. Results indicate the presence of the IAM pathway (Trp → IAM → IAA) and the possible presence of additional pathways for IAA biosynthesis in *Streptomyces*.**

**Keywords:** *Streptomyces* spp., indole-3-acetic acid, tryptophan, indole-3-acetamide pathway

## INTRODUCTION

The ability to produce the plant hormone indole-3-acetic acid (IAA) is widespread among micro-organisms that are commonly associated with plant surfaces (Wichner & Libbert, 1968). IAA has been detected in plant pathogenic and non-pathogenic bacteria (Fett *et al.*, 1987; Manulis *et al.*, 1991; Badenoch-Jones *et al.*, 1982; Tien *et al.*, 1979; Barea & Brown, 1974) as well as in mycorrhizal and other fungi (Ek *et al.*, 1983; Gruen, 1959). IAA has been shown to be essential for gall formation by *Pseudomonas syringae* pv. *savastanoi* (Comai & Kosuge, 1983) and *Agrobacterium tumefaciens* (Liv *et al.*, 1982) and root induction by *Agrobacterium rhizogenes* (Offringa *et al.*, 1986). Production of IAA from L-tryptophan (Trp) with indole-3-acetamide (IAM) as an intermediate (see Fig. 4) has been detected in gall-forming phytopathogenic bacteria (Van Onckelen *et al.*, 1986; Comai & Kosuge, 1983; Manulis *et al.*, 1991), in *Bradyrhizobium* spp. (Sekine *et al.*, 1988) and it may occur in other bacteria also (Fett *et al.*, 1987). Although the IAM pathway has been recently demonstrated in higher plants (Kawaguchi *et al.*, 1993; Saotome *et al.*, 1993) its presence in plants might be rare. In contrast to the IAM pathway,

the indolepyruvic acid (IPvA) route is quite common in many microbes and plants (e.g. Fett *et al.*, 1987; Iskrac, 1984).

Production of IAA by *Streptomyces* spp. has been reported previously (El-Shanshoury, 1991; El-Sayed *et al.*, 1987; Hirata, 1959) although its identity was not rigorously confirmed by modern analytical methods. It has been suggested that IAA may act as an endogenous regulator of spore germination in *Streptomyces atroolivaceus* (El-Shanshoury, 1991) and may be involved in differentiation of actinomycetes (Efremenkova *et al.*, 1985). *Streptomyces scabies* (Hooker, 1981) as well as other *Streptomyces* spp. (Doering-Saad *et al.*, 1992) have been reported as the causal agents of potato scab disease. The observation that development of potato scab formation is associated with cell division during development of wound periderm (Lapwood, 1973) may lead to the hypothesis that IAA is involved in pathogenicity of *Streptomyces* spp. The present study was undertaken to demonstrate the production of IAA by plant pathogenic and non-pathogenic *Streptomyces* strains and to characterize its biosynthetic pathway.

## METHODS

**Micro-organisms and growth conditions.** The *Streptomyces* strains used in this study were as follows: *S. violaceus* (44, NP-211, NP-214) and *S. griseus* (20, 26, 42), isolated from scabby

**Abbreviations:** IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; IAM, indole-3-acetamide; IEt, indole-3-ethanol; ILA, indole-3-lactic acid; IPvA, indole-3-pyruvic acid; Trp, tryptophan.

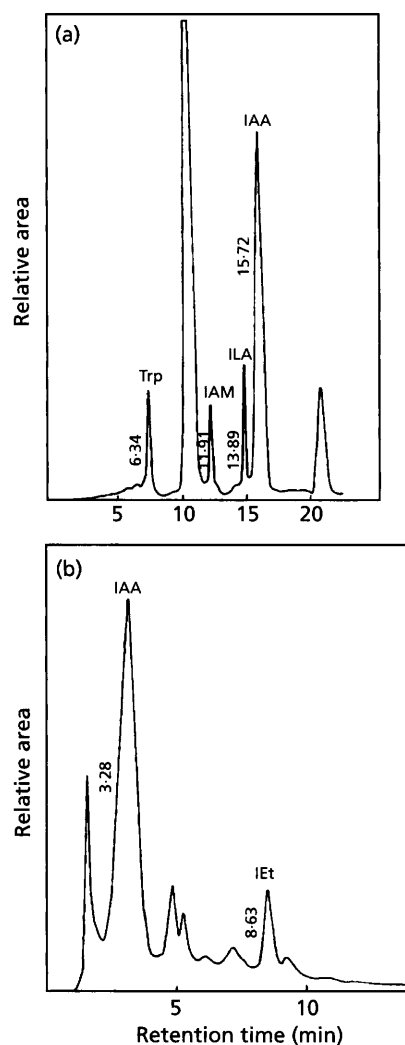
potatoes in Israel; *S. scabies* (RL 840170) and *S. exfoliatus* (RL 830103), obtained from R. Loria (Department of Plant Pathology, Cornell University, Ithaca, NY, USA). The identification of these strains according to numerical taxonomy was described by Doering-Saad *et al.* (1992). *S. coelicolor* M110 and *S. lividans* 1326 were obtained from H. Schrempf (Fachbereich Biologie/Chemie, Universität Osnabrück, Osnabrück, Germany). Strains were grown at 28 °C on R2 agar medium without sucrose and maintained in glycerol at -70 °C as described by Hopwood *et al.* (1985).

**Production and extraction of IAA and other indole derivatives.** The routes for IAA biosynthesis from Trp were investigated by feeding a concentrated bacterial cell suspension with Trp or other indole derivatives and determining the metabolic intermediates released into the supernatant. Bacteria were grown in 1 l Erlenmeyer flasks containing 200 ml tryptic soy broth (TSB) (Hopwood *et al.*, 1985) supplemented with 200 mg Trp l<sup>-1</sup>. The cultures were grown on a rotary shaker at 28 °C for 7–10 d. The cells were then centrifuged from the culture medium at 10000 g for 15 min at 4 °C, washed with 0.01 M phosphate buffered saline (PBS), pH 7.2 and resuspended in the feeding solution. The experiment was carried out in 250 ml Erlenmeyer flasks containing fresh cells (25 g) and feeding solution composed of: PBS, 2 mM; Tricine/KOH buffer (Sigma) pH 8.3, 30 mM; D-glucose, 2.5 mM; MgSO<sub>4</sub>, 0.2 mM; streptomycin, 50 µg ml<sup>-1</sup>; penicillin 50 µg ml<sup>-1</sup>, cetyltrimethylammonium bromide (CTAB), 2.7 mM; and Trp, 7.6 mM, or other indole derivatives (2 mM), in a total volume of 90 ml. The viability of the bacterial cells was not affected by addition of the CTAB. All the chemicals and flasks were sterilized and the reaction mixture was prepared under aseptic conditions. Flasks were incubated at 28 °C for 3 d, in the dark on a rotary shaker, prior to the removal of the cells by centrifugation at 10000 g for 15 min.

The supernatant was acidified to pH 3.0 with 2 M HCl and extracted twice with equal volume of analytical ethyl acetate. The ethyl acetate extracts were pooled and flash-evaporated at 30 °C. The dry pellet was dissolved in 500 µl methanol containing 1% butylated hydroxytoluene (BHT) and chromatographed on a silica gel 60H column (2 × 10 cm). The column was washed three times with each of the following fractions of hexane/ethyl acetate (v/v): (I) 38:12, (II) 51:24, (III) 45:30, (IV) 33:42, (V) 21:54 and (VI) 12:60 with three parts of methanol and twice with 25 ml of methanol. The various fractions of each solvent were combined, reduced to dryness *in vacuo* and dissolved in 300 µl methanol containing 1% BHT. The resultant solutions were analysed for the presence of indole metabolites. IAA and IET were eluted in fraction I, indole lactic acid (ILA) was eluted in fraction IV and IAM and Trp were eluted in fraction VI.

**Identification of indole metabolites.** Initial identification of IAA and other indole derivatives was carried out by thin-layer chromatography (TLC) on 0.25 mm glass plates with authentic standards in either acidic or basic solvents: (A) chloroform/ethyl acetate/formic acid (35:55:10, by vol.); (B) ethyl acetate/propan-2-ol/ammonia (45:35:20, by vol.). The plates were sprayed with Ehmann's reagent (Ehmann, 1977) followed by heating and washing with tap-water for intensification of the stained spots. Identification of IPvA and IAAlid was carried out as described by Manulis *et al.* (1991).

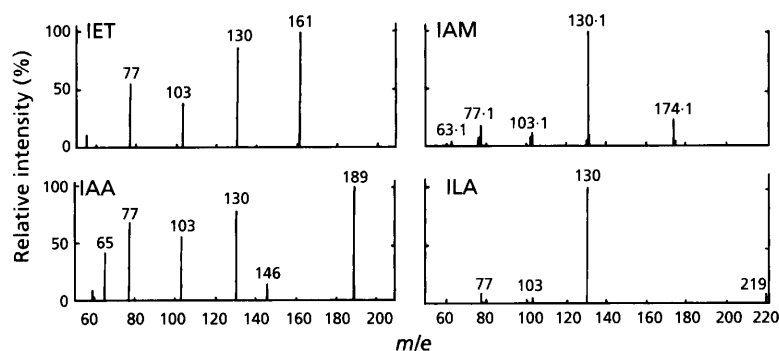
Quantitative analysis of the various indoles was performed by high performance liquid chromatography (HPLC) in a Varian 5000 chromatograph with a Rheodyne 7125 sample injector (50 µl loop), a Varian UV-100 detector at 280 nm, and a Hewlett-Packard 3390A integrator. The indole compounds were applied



**Fig. 1.** Reverse-phase HPLC profile of indole derivatives produced by *S. violaceus* strain 44. (a) Ethyl acetate extract of Trp-feeding solution was chromatographed on a silica gel 60H column as described in Methods. Eluted fractions I, IV and VI were combined, reduced to dryness and dissolved in 300 µl methanol. Following injection (50 µl), the column was eluted with a gradient of 10–90% (v/v) methanol containing 0.1% acetic acid. (b) The same as (a) except that only fraction I (containing IAA and IET) from the silica gel 60H column was injected and a gradient of 25–75% methanol containing 20 mM ammonium acetate, pH 6.5 was used.

to a Whatman ODSRP18 (5 µm) column and eluted with a 35 min gradient of 10–90% (v/v) methanol containing 0.1% acetic acid. Solvents were delivered at a flow rate of 1.5 ml min<sup>-1</sup>. This solvent could separate IAA, IAM, ILA and Trp. Separation of IET from IAA was achieved by employing a 20 min gradient of 25–75% methanol (v/v) containing 20 mM ammonium acetate buffer, pH 6.5. Combined gas chromatography-mass spectrometry (GC-MS) was performed on a Finnigan MAT 4600 mass spectrometer as described by Manulis *et al.* (1991). The indole derivatives were purified by HPLC prior to GC-MS analysis.

**Incorporation of labelled tryptophan into indole metabolites.**  
 L-[3-<sup>14</sup>C]Tryptophan [55.4 mCi mmol<sup>-1</sup> (2.05 GBq mmol<sup>-1</sup>)]



**Fig. 2.** Electron-impact mass spectra of the IET, IAM, methyl IAA and ILA peaks obtained from HPLC (Fig. 1). The mass spectra presented are identical with those of authentic samples.

was obtained from Amersham, and used for feeding experiments. Feeding of labelled Trp to bacterial cells was as described above except that only radioactive Trp was added at a final concentration of 1.6 mM instead of 7.6 mM.

Identification of radioactive intermediates was based on co-chromatography with authentic standards using the acidic and basic TLC solvents described above. The non-fluorescent TLC plates were dried and autoradiography was carried out at room temperature with Kodak XAR-5 film for 72 h.

## RESULTS

### Identification of indole derivatives

Characterization of the indole metabolites was initially performed by co-chromatography of the ethyl acetate extract with authentic compounds on TLC in solvents A and B. After the plates had been sprayed with Ehrmann's reagent, IAA, IAM, ILA, IET and Trp appeared in solvent A as blue spots with  $R_F$  values of 0.75, 0.61, 0.5, 0.7 and 0.04, respectively, and in solvent B with  $R_F$  values of 0.41, 0.92, 0.42, 0.95 and 0.32, respectively. Attempts to detect IPvA and IAAld following their conversion into 2,4-dinitrophenylhydrazones and chromatography on TLC gave negative results.

**Table 1.** Release of indole metabolites by cell suspensions of *Streptomyces* spp.

Concentrated cell suspensions were fed with tryptophan (7.6 mM) under sterile conditions and the indole derivatives were extracted from the supernatant as described in Methods. Quantitative determination of the indole derivatives was carried out by HPLC. The concentration ranges of the various compounds indicating the minimum and maximum amounts were obtained from four different experiments.

Indole derivative	Concentration detected ( $\mu\text{g ml}^{-1}$ )	
	<i>S. violaceus</i> (44)	<i>S. exfoliatus</i> (RL 830103)
Indole-3-acetic acid	10–30	5–10
Indole-3-acetamide	10–60	5–10
Indole-3-ethanol	10–30	5–20
Indole-3-lactic acid	10–50	10–50

Further identification was achieved by fractionation of the various compounds on a silica gel column and injection of each fraction into an HPLC apparatus. Results presented in Fig. 1(a) show the detection of Trp, IAM, ILA and IAA with retention times of 6.34, 11.91, 13.89 and 15.72 min, respectively, when the acidic methanol gradient was employed. Two other major peaks remained unidentified. Since IAA and IET showed identical retention times with the acidic solvent, a basic methanol solvent was used for separating these compounds (Fig. 1b). The retention times for IAA and IET were 3.28 and 8.63 min, respectively. The mass spectra obtained from GC-MS for the HPLC peaks of IAA, IAM, ILA and IET are illustrated in Fig. 2. They were identical to those obtained for authentic compounds. Quantitative estimation of the identified indole derivatives produced by *S. violaceus* strain 44 and *S. exfoliatus* strain RL 830103 after the cells were fed with Trp is summarized in Table 1. The presence of a significant concentration of IAM and IAA in both species could suggest the existence of the IAM pathway (see Fig. 4).

### Metabolism of indole intermediates by cell suspensions

The ability of concentrated bacterial cells to metabolize infiltrated indole intermediates into IAA could provide evidence for the presence of a metabolic pathway for IAA biosynthesis. These experiments were performed by permeating the cells with various indole derivatives and detecting the release of IAA into the supernatant. Results presented in Table 2 indicate that all the tested indole intermediates, namely IAM, IET, ILA and IAAld were converted into IAA when incubated in the presence of cells of *S. violaceus* or *S. exfoliatus*. IPvA was not included in these experiments since a significant breakdown into IAA occurred even in the absence of bacterial cells. The traces of IAA in the supernatants of unfed cells (Table 2) probably originated from their internal IAA pool, which had accumulated during their prior growth in TSB medium supplemented with Trp.

### Metabolism of radioactive tryptophan into indole metabolites

The results of feeding *S. violaceus* strain 44 with L-[3- $^{14}\text{C}$ ]tryptophan (Fig. 3) indicate incorporation of radioactivity into IAM, IAA and IAL. IET, which had

**Table 2.** Metabolism of indole derivatives into IAA by *S. violaceus* and *S. exfoliatus*

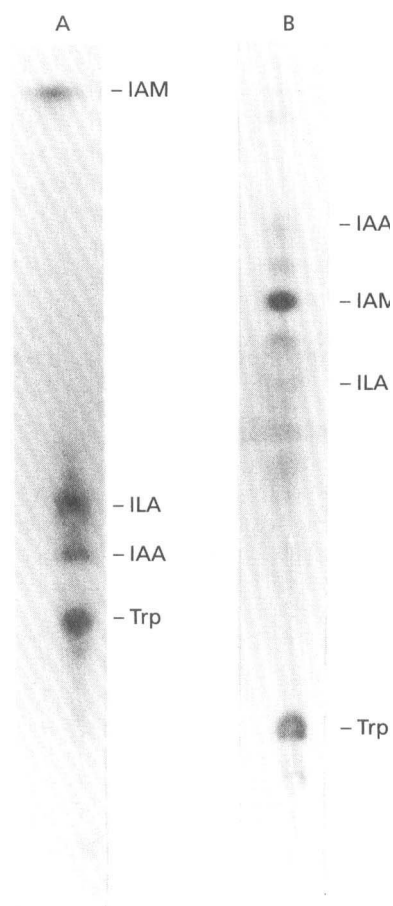
Concentrated cell suspensions were fed for 72 h with various indole derivatives (2 mM) under sterile conditions and IAA was extracted from the supernatant and measured as described in Methods. Results presented were obtained from three different experiments.

Compound added	IAA concentration ( $\mu\text{g ml}^{-1}$ )	
	<i>S. violaceus</i> (44)	<i>S. exfoliatus</i> (RL 830103)
Indole-3-acetamide	11–17	10–20
Indole-3-ethanol	10–25	10–20
Indole-3-lactic	10–40	10–40
Indole-3-acetaldehyde	10–25	10–30
No addition	3–5	3–5

**Table 3.** Production of IAA by various *Streptomyces* spp.

Estimation of IAA was performed as described in Table 2. (+) indicates an IAA level of above  $5 \mu\text{g ml}^{-1}$  in two different experiments. Characterization of the various *Streptomyces* strains and their pathogenicity was according to Doering-Saad *et al.* (1992).

<i>Streptomyces</i> sp.	Strain	Pathogenicity on potato	IAA production
<i>S. violaceus</i>	44	+	+
	NP 214	–	+
	NP 211	–	+
<i>S. scabies</i>	RL 840170	+	+
	20	+	+
<i>S. griseus</i>	26	+	+
	42	+	+
	RL 830103	+	+
<i>S. exfoliatus</i>	RL 830103	+	+
<i>S. coelicolor</i>	S.MII0	–	+
<i>S. lividans</i>	1362	–	+



**Fig. 3.** Autoradiography of thin-layer chromatograph of indole derivatives produced by *S. violaceus* (strain 44) following incorporation of  $[^{14}\text{C}]\text{Trp}$ . Experimental conditions are described in Methods. A and B represent two separate experiments. In A, the chromatogram was developed in the basic solvent, in B with the acidic solvent.

previously been identified by GC-MS (Fig. 2), was not detected as a labelled compound under the experimental conditions used. A similar incorporation pattern of radioactive Trp into indole derivatives was obtained with *S. exfoliatus* (RL 830103).

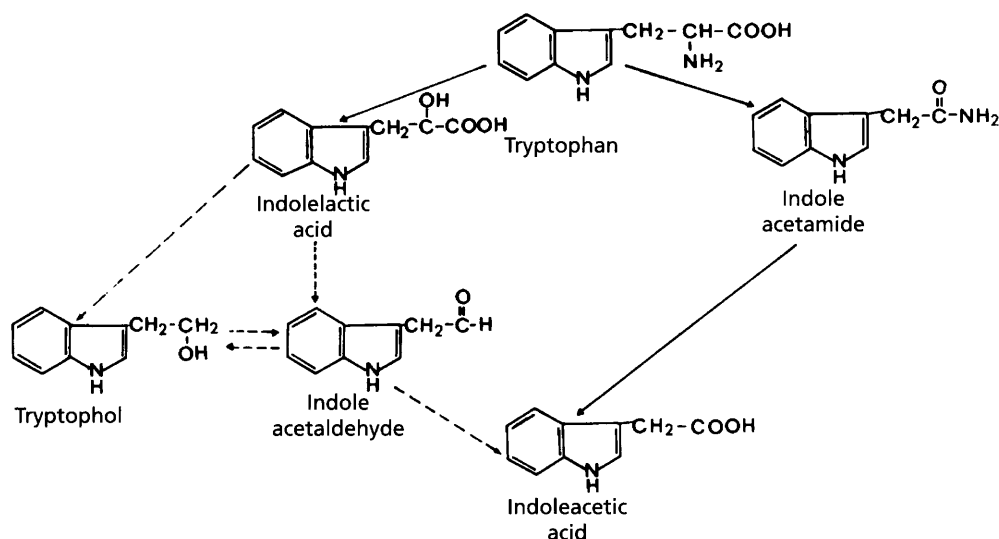
#### Production of IAA by various pathogenic and non-pathogenic *Streptomyces* strains

Results presented in Table 3 suggest that IAA biosynthesis from Trp is widespread among different *Streptomyces* spp. and is not exclusive to strains which cause potato scab.

#### DISCUSSION

The ability of *Streptomyces* spp. to produce IAA in the presence of Trp may result from several biosynthetic pathways. The presence of the IAM pathway in *S. violaceus* and *S. exfoliatus* is supported by the following observations: (i) chemical identification of the indole intermediate, IAM, during catabolism of Trp into IAA; (ii) production of IAA following infiltration of the bacterial cells with IAM; and (iii) incorporation of radioactive carbon from Trp into IAM and IAA. This pathway appears to be prevalent in bacteria but seems to be quite rare in higher plants. It has been suggested (Manulis *et al.*, 1991; Iskrac, 1984) that by utilizing the IAM pathway pathogenic bacteria may override the regulatory mechanism through which plants that lack this pathway control the IAA level. In addition, the IAM pathway can detoxify several growth-inhibiting tryptophan analogues (Kosuge *et al.*, 1985).

The detection of ILA and IET and the ability of the *Streptomyces* cells to convert them into IAA may indicate the existence of additional pathways for IAA biosynthesis. These intermediates were also identified in other microbes



**Fig. 4.** Indoleacetamide pathway for IAA biosynthesis and other indole derivatives identified in *S. violaceus* during tryptophan metabolism. With the exception of IAald all the other indole derivatives were identified. Solid lines indicate incorporation of radioactivity from Trp into indole derivatives whereas dashed lines signify hypothetical routes for transformation of the indole compounds into IAA.

and plants, such as *Frankia* sp. (Berry *et al.*, 1989), *Agrobacterium tumefaciens* (Kutacek & Rovenska, 1990) and tomato (Gibson *et al.*, 1972). In the latter, it has been proposed that ILA might act as precursor of IAA through a pathway initially involving decarboxylation of the lactic acid to produce IET (Gibson *et al.*, 1972). The IET could then be oxidized to IAald and IAA (Fig. 4). A similar pathway may exist in *Streptomyces*, and it could explain the detection of the two compounds. An alternative possibility is the existence of the IPvA pathway which has been reported in other bacteria (Manulis *et al.*, 1991; Kutacek & Rovenska, 1990). Even though we failed to detect the intermediates IPvA and IAald, we did detect ILA, which is a common product of IPvA reduction, and IET, which is a common product of IAald reduction. It has been speculated that in micro-organisms, ILA has the characteristics of a secondary biologically inactive detoxification product of indole metabolism (Kutacek & Rovenska, 1990). This might also explain the production of ILA during Trp metabolism in *Streptomyces*. In the [ $^{14}\text{C}$ ]Trp incorporation experiments we could detect radioactivity in ILA but not in IET, in spite of the fact that IET was identified by GC-MS. It is possible that IET is rapidly oxidized to IAald so that it could be detected only when an excessive concentration of Trp was given to the cells. It should be pointed out that the final concentration of Trp in the feeding solution of the radioactive experiment was 1.6 mM as compared to 7.6 mM in the non-radioactive experiment.

Strains representing six different *Streptomyces* spp. were found to be capable of induced synthesis of IAA in the presence of Trp. Thus IAA production may be widespread among streptomycetes, which are often associated with plant roots or tubers. The possible relationship of IAA secretion to pathogenicity of *Streptomyces* strains on

potato has yet to be proven by studying the pathogenicity of an IAA-lacking mutant. However, IAA might exhibit additional effects on plant tissue which could benefit *Streptomyces* multiplication *in planta*. Thus, exogenous IAA can induce plant-cell-wall loosening and membrane leakiness thereby stimulating the exudation of nutrients (Kudent & Libbert, 1972). IAA-producing bacteria might therefore have an advantage over non-producing bacteria in acquisition of nutrients required for growth. It has been suggested that auxin and cytokinin concentration in plant tissues invaded by pathogens could affect the levels of hydrolytic enzymes such as chitinases and  $\beta$ -1,3-glucanases, which are thought to be involved in plant defence responses (Shinshi *et al.*, 1987). IAA was also found to suppress the hypersensitive response of host plants (Robinnete & Matthyse, 1990). Thus IAA might alter plant defence responses, as a step toward optimizing the microbial interaction with host plants.

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